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# Effect of Macrophage Depletion on Immune Effector Mechanisms during Corneal Allograft Rejection in Rats

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**PURPOSE.** In rats, corneal allograft rejection is delayed for at least 100 days by clodronate liposomes. These liposomes selectively deplete macrophages. To investigate the immunologic basis for absence of graft rejection in treated rats, the effect of these liposomes on the generation of cytotoxic T lymphocytes (CTLs) and antibody production after orthotopic corneal allotransplantation was determined.

**METHODS.** Transplantations of corneal buttons from PVG rats were performed in AO rats. After surgery, one group received clodronate liposomes subconjunctivally at five time points, and the other group remained untreated. On postoperative day (POD) 3, 7, 12, or 17, rats were killed, the presence of CTLs was investigated at three different anatomic locations, and antibodies against donor antigens were tested.

**RESULTS.** No significant differences were found between the groups tested 3 and 7 days after surgery. But on POD 12 (the time of onset of rejection in the untreated group) and on POD 17, the CTL activities detected in the submandibular lymph nodes ( $P \leq 0.008$ ) and the spleen ( $P \leq 0.009$ ) were significantly less in the treated groups compared with the untreated groups. In the untreated groups complement-independent antibodies were present only on POD 17, whereas no antibodies were found in the treated rats.

**CONCLUSIONS.** Local treatment with clodronate liposomes was shown to downregulate local and systemic CTL responses and to prevent the generation of antibodies. Local depletion of macrophages in the initiation phase of the immune response appears to lead to a less vigorous attack on the grafted tissue and therefore to promote graft survival. (*Invest Ophthalmol Vis Sci.* 2000;41:2239-2247)

As a remedy for corneal defects, corneal transplantation appears to be very effective. Therefore, each year a large number of corneal transplantations is performed, with an estimated 3-year failure rate at 10% to 40%.<sup>1-3</sup> The major cause for corneal graft failure is immunologic rejection of foreign tissue. The purpose of topical or systemic administration of corticosteroids is to suppress this immune response, but it is not always efficient. To improve the outcome of high-risk corneal grafts in particular, research is performed to find new therapies that are more effective than those currently used. Promising studies have been performed using oral immu-

nization,<sup>4</sup> blocking of particular cytokines or adhesion molecules,<sup>5-7</sup> or local blocking with anti-CD4.<sup>8</sup>

Histopathologic studies of corneal graft rejection show an influx of not only T lymphocytes but also many macrophages.<sup>9</sup> Although the current opinion is that rejection is mediated by T lymphocytes, Van der Veen et al.<sup>10</sup> performed a study to determine the contribution of the macrophage to immunologic corneal graft rejection in high-risk rats. Using dichloromethylene diphosphonate (clodronate)-containing liposomes, macrophages could be selectively depleted. Subconjunctival administration of these liposomes after orthotopic corneal transplantation to recipient rats with a history of corneal inflammation, surprisingly resulted in complete graft survival in all treated rats for a follow-up period of 100 days, whereas the control group rejected the grafts between 12 and 17 days after surgery. Therefore, macrophages also seem to play an important role in corneal graft rejection.

It remains to be determined how the clodronate liposomes interfered in the rejection process. With regard to the various functions of macrophages, they can be involved in both the afferent and the efferent arc of the immune response leading to graft rejection. Through processing and presentation of foreign antigen to T lymphocytes they can participate in the afferent arc, but they are also able to play the role of effector cells and destroy the graft.<sup>11,12</sup>

In this study, we used a different rat strain combination without prior induction of keratitis to validate the clodronate

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liposome treatment. To unravel the immunologic basis for the absence of graft rejection in clodronate liposome-treated rats we used this strain combination to measure the cytotoxic T lymphocyte (CTL) and antibody responses from postoperative days (PODs) 3 to 17.

## METHODS

### Animals

Inbred male AO/OlaHsd (RT1<sup>b</sup>) and PVG/OlaHsd (RT1<sup>c</sup>) rats were obtained from Harlan UK (Bicester, UK). At the time of transplantation, AO recipients and donor PVG rats were 10 to 12 weeks of age. The animal studies were approved by the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences and conformed to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Anesthesia

Donor rats were killed by an intracardial injection of pentobarbital (0.5 ml; Euthesate; Apharmo, Arnhem, The Netherlands). Recipients were anesthetized by an intramuscular injection of a mixture of fluanisone and fentanyl citrate (0.5 ml/kg; Hypnorm; Janssen Pharmaceutica, Beerse, Belgium) and an intraperitoneal injection of diazepam (2.5 mg/kg). Oxybuprocaine hydrochloride (0.4%) was used for topical anesthesia of the cornea.

### Orthotopic Corneal Transplantation

Full-thickness corneal transplantations were performed in the right eye of the recipient rat using a technique previously described.<sup>10</sup> Briefly, before surgery dilation of the iris was obtained by subcutaneous injection of atropine sulfate (0.15 mg/kg) and topical administration of atropine 1% sulfate and 10% phenylephrine hydrochloride. With a 3-mm trephine and curved Vannas scissors, the corneal button was removed from the donor and recipient rats. The donor cornea was stored in Eagle's modified essential medium with 2% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) until use. Eight stitches were made using a continuous 10-0 monofilament nylon suture (Alcon, Fort Worth, TX) to secure the donor button into the recipient graft bed. After surgery, no attempt was made to re-form the anterior chamber or to remove the suture.

### Clinical Evaluation

Grafts were observed for occurrence of graft rejection using an operating microscope, on each alternate day until POD 14 and once a week thereafter. Rejection was diagnosed as an opacity score of 3 or higher in a previously clear graft. Opacity was scored from 0 to 4. A score of 3 meant that the iris vessels were not visible, but the pupil margins could be distinguished.<sup>9</sup> Corneal transplantations that were complicated by technical failures were excluded from the study, as well as grafts that had an opacity score of 3 or higher during clodronate liposome injections (see experimental design).

### Preparation of Clodronate Liposomes

Clodronate liposomes were prepared as has been described.<sup>13</sup> In short, 75 mg phosphatidylcholine and 11 mg cholesterol

were dissolved in chloroform and a thin lipid film was produced by low-vacuum rotary evaporation. This film was dispersed in 10 ml phosphate-buffered saline (PBS) solution in which 1.8 to 1.9 ml dichloromethylene diphosphonate (clodronate [Cl<sub>2</sub>MDP]; the kind gift of Boehringer Mannheim, Mannheim, Germany) was dissolved. The suspension was maintained at room temperature for 2 hours followed by sonication for 3 minutes in a water bath sonicator. After another 2 hours at room temperature, the dichloromethylene diphosphonate liposome suspension was centrifuged at 100,000g for 30 minutes to remove free dichloromethylene diphosphonate. The liposomes were resuspended in 4 ml PBS and stored at 4°C until use.

### Experimental Design

Ninety-one transplantations were performed of which five were excluded: four because of technical failures of the transplantations, such as extensive synechiae and hyphema, and one because of an anesthesia problem. Four of the transplantations were autografts. The allografted animals were divided into two experimental groups: an untreated and a clodronate liposome-treated group. The latter received subconjunctival injections of clodronate liposomes immediately after transplantation and on PODs 2, 4, 6, and 8. At each time point 100 µl of these liposomes was injected near the limbus, dispensed over four locations (±25 µl each), to achieve an equal distribution around the cornea. Previously, it had been demonstrated that during clodronate liposome injections the graft became slightly more opaque than the untreated grafts.<sup>10</sup> It was found that an opacity score of three or higher was not acceptable, because the graft will not regain clarity after POD 8. For this reason, 3 of the 41 clodronate liposome-treated animals had to be excluded from the study because the graft rejection time could not be determined. In these rats, failure was thought to be due to mechanical reasons, such as liposome clotting or increased pressure around the eyeball. An immunologic cause for rejection seems improbable so soon after grafting in an avascular cornea and had never occurred this early in the untreated group.

**Experiment 1.** Twenty-one grafts were observed for the appearance of graft rejection for 100 days. The four autografts in AO recipients were used to test for the correct transplantation technique and the nine untreated allografts to determine the rejection time. Eight allogeneic grafts were treated with clodronate liposomes to determine whether this treatment also prevents graft rejection in the PVG-AO rat strain combination.

**Experiment 2.** To determine the immunologic basis for the absence of graft rejection after clodronate liposome treatment, rats were killed at four different time points. Two time points were chosen before rejection occurred in the untreated allogeneic group (PODs 3 and 7), one at the onset of graft rejection in the untreated allogeneic group (POD 12) and approximately one 5 days after rejection (POD 17). Lymphoid cells were isolated from three locations to determine the presence of CTLs: the submandibular lymph nodes (SLNs), the mesenteric lymph nodes (MLNs), and the spleen. To analyze for the presence of alloantibodies, serum was obtained at these same time points. All serum samples were heat-inactivated and stored at -20°C in small aliquots until use.

**Experiment 3.** To exclude the possibility that local administration of clodronate liposomes suppresses the whole

immune system, five AO rats were immunized intraperitoneally with PVG lymphocytes. Three of them were treated with subconjunctival injections of clodronate liposomes on the day of immunization and 2, 4, 6, and 8 days later; the other two rats remained untreated. Ten days after immunization, rats were killed, and the presence of CTLs and antibodies directed toward PVG lymphocytes was tested in a chromium-release assay and an immunofluorescence assay.

### Cell-Mediated Cytotoxicity Assay

The specific cytotoxic activity of lymphoid cells derived from grafted rats was determined by a chromium 51 release assay. Cells isolated from the SLNs, MLNs, and spleen were measured separately. Single-cell suspensions were prepared by gently teasing the tissue through a nylon-mesh strainer (70- $\mu$ m Cell Strainer; Falcon, Becton Dickinson, Franklin Lakes, NJ). The cell suspensions were washed twice and resuspended in culture medium (RPMI 1640 containing 2.0 mM L-glutamine, 10 mM HEPES buffer [Life Technologies], 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 10% heat-inactivated fetal calf serum [Life Technologies], and  $3 \times 10^{-5}$  M 2-mercaptoethanol [Sigma, St. Louis, MO]) at a concentration of  $5 \times 10^6$  cells/ml. Thymocytes derived from a young naive PVG rat were prepared as described and irradiated (30 Gy) with a cesium 137 source. These irradiated stimulator cells were adjusted to a cell concentration of  $5 \times 10^6$  cell/ml; 0.5 ml of the suspension was mixed with 1 ml effector cells in 24-well trays (Costar, Cambridge, MA), total volume 1.5 ml; and cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After 5 days the effector cells from six identical wells were pooled, and cell viability was determined by trypan blue exclusion. Cells were centrifuged at 200g for 10 minutes and resuspended in culture medium at the following concentrations:  $10 \times 10^6$ /ml;  $5 \times 10^6$ /ml;  $2.5 \times 10^6$ /ml and  $1.25 \times 10^6$ /ml.

SLNs and MLNs from a naive PVG rat served as target cells. Target cells were cultured for 5 days in culture medium at a concentration of  $5 \times 10^6$  cells/ml, and after 2 days in culture concanavalin A (ConA 2  $\mu$ g/ml; Life Technologies) was added. On the day of the assay, target cells were washed and labeled with 100  $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham, Little Chalfont, UK) at 37°C for 1 hour. Labeled cells were washed three times with culture medium, incubated for 30 minutes in 20 ml culture medium to remove spontaneously released <sup>51</sup>Cr, centrifuged (10 minutes, 200g) and resuspended at a concentration of  $2 \times 10^5$  cells/ml. Fifty microliters of labeled target cells ( $10^4$  cells) were plated into wells of round-bottomed 96-well microtiter plates (Costar). One hundred microliters of effector cells in different concentrations was added in triplicate to the target cells. Effector-to-target ratios ranged from 100:1 to 12.5:1. Plates were centrifuged at 100g for 3 minutes, followed by a 4-hour incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After 4 hours, the plates were centrifuged for 10 minutes at 500g and 100  $\mu$ l supernatant was removed from each well for counting of radioactivity in a spectrometer (Auto-gamma; Packard, Meriden, CT). The cytotoxicity of each sample was determined as follows:

Percentage specific <sup>51</sup>Cr release

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Spontaneous release was determined by counting supernatants from wells containing target cells and culture medium only. The maximum release was determined by counting the supernatant of wells containing target cells and 1% Triton X-100 (Sigma). The CTL assays were tested each time for reproducibility with cell suspensions derived from the SLNs, MLNs, and spleen of naive AO rats and of AO rats that had been immunized against alloantigens by repeated intraperitoneal injections with PVG lymphocytes and splenocytes.

### Assay of Alloantibodies in Serum

The presence of specific antibodies against PVG cells was tested by indirect immunofluorescence assay. PVG lymphocytes were isolated and cultured for 3 days in the presence of ConA (2  $\mu$ g/ml) to increase their size and enhance their major histocompatibility complex (MHC) expression. After 3 days, the cells were washed and fixed with 2% paraformaldehyde. Subsequently, these fixed cells were washed twice in washing medium (PBS, 1% bovine serum albumin [Sigma], and 0.2% sodium azide), centrifuged for 10 minutes at 200g, and resuspended at  $5 \times 10^6$  cells/ml. Ten microliters serum was added to 90  $\mu$ l of these cells. After an incubation for 1 hour, cells were washed and incubated for another hour at 4°C with a 1:50 dilution of rabbit anti-rat Ig antibody labeled with fluorescein isothiocyanate (Rara-Ig-FITC; Nordic, Tilburg, The Netherlands). Cells were washed once, 1 drop of Evans blue was added to the pellet, and cells were washed again. Finally, they were suspended in PBS-glycerol, a drop of Vectashield (Vector; Burlingame, CA) was added, and they were mounted on a glass slide with a coverslip. The cells were scored for the presence of immunofluorescence-positive cells by a masked observer, and the percentage of positive cells was calculated. In each assay, sera from grafted rats and samples from a pool of naive AO rats or from a pool of AO rats immunized against PVG antigens were included.

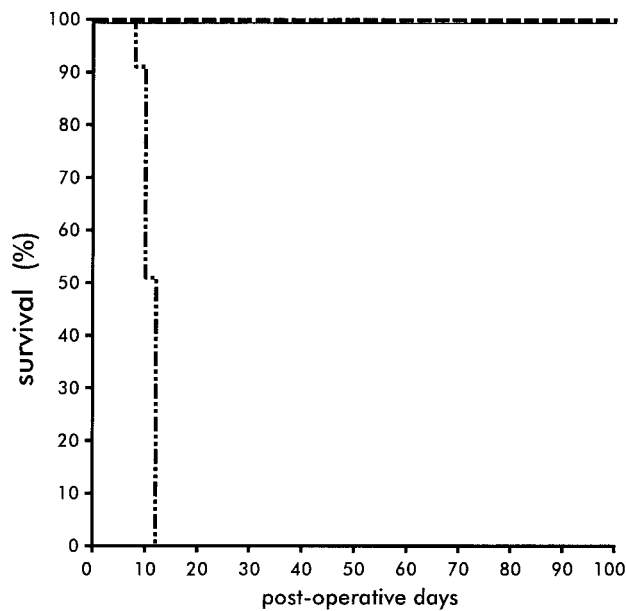
### Assay of Complement-Dependent Alloantibody in Serum

To test the specific cytotoxic activity of the serum samples a chromium release assay was used. The Cr 51-labeled target cells were prepared as described. Fifty microliters of these cells ( $5 \times 10^6$  cells/ml) was plated into wells of a round-bottomed 96-well plate and incubated at 37°C for 60 minutes with 50  $\mu$ l serum dilution (1:10, 1:20, 1:40, and 1:80) obtained from graft-recipient rats. Subsequently, 50  $\mu$ l fresh normal rat serum was added, as a source of complement, and the incubation continued for 60 minutes. The plates were then centrifuged at 500g for 10 minutes, and 100  $\mu$ l of the supernatant was removed and counted in a gamma counter. The percentage of specific release of serum samples was determined as described. In each assay, a serum sample from a pool of naive AO rats and a serum sample from a pool of AO rats that had been immunized against PVG antigens were tested as a standard.

### Statistical Analysis

The graft survival curves were compared using the log rank test. Statistical analysis of the cytotoxic and immunofluorescence assays was performed using the Mann-Whitney test. The two groups were compared at the effector-to-target ratio of 100 to 1 and serum dilution of 1:10. The statistical analyses were





**FIGURE 1.** Survival curves of 82 orthotopic corneal grafts in AO rats. Three experimental groups were distinguished: AO rats receiving autografts (*dotted line*); untreated allografted AO rats (*dashed-dotted line*); and clodronate liposome-treated allografted AO rats (*solid line*). A significant difference was found ( $P < 0.001$ ) between the clodronate liposome-treated allografts and the untreated allografts.

corrected for multiple comparisons;  $P < 0.03$  was considered statistically significant.

## RESULTS

### Fate of Orthotopic Corneal Grafts

Eighty-two orthotopic corneal grafts were observed for occurrence of graft rejection (Fig. 1). The 21 rats of experiment 1 were evaluated for the follow-up period of 100 days, whereas the 61 rats used in experiment 2 were evaluated until they were killed at POD 3, 7, 12 or 17.

All four autografts survived for at least 100 days. In the 40 untreated rats with a graft, no graft survival was observed beyond 12 days (mean survival time of 10.9 days). Of the 38 allogeneic corneal grafts that were treated with clodronate liposomes, not a single graft rejection was observed. In the eight clodronate liposome-treated rats of experiment 1 graft survival was prolonged beyond 100 days. In comparing the survival times of the two groups, a significant difference of  $P < 0.001$  was found.

### CTL Activity against Donor Antigens in Recipients of Corneal Allografts

In experiment 2, at four time points after orthotopic allotransplantation, untreated AO rats and clodronate liposome-treated AO rats were killed to determine the presence of CTLs against donor PVG cells. Cells were isolated from three locations to determine differences in local and systemic CTL activity between the two groups. Local CTL activity was determined by taking cells derived from the SLNs (Fig. 2). Comparing the two treatment groups at the effector-to-target ratio of 100 to 1, no

difference was noted on PODs 3 and 7. But at time of the onset of graft rejection in the untreated group, POD 12 and 5 days later, a significant difference was noted ( $P = 0.006$  and  $P = 0.008$ , respectively). Far higher CTL activity was found in the untreated rats than in the clodronate liposome-treated rats.

The second more remote location investigated for presence of CTLs was the MLNs. No difference was detected at any of the four postoperative time points (Fig. 3). The spleen was assayed for presence of systemic CTL activity, and a pattern similar to that in the local lymph nodes was found (Fig. 4). Before graft rejection in the untreated group, no activity was present, but at the time of graft destruction, significantly more CTL activity was present in the spleen of untreated rats than in treated rats. A difference was demonstrated at POD 12 ( $P = 0.008$ ) and later at POD 17 ( $P = 0.009$ ).

### Alloantibody Response against Donor Antigens in Recipients of Allografts

The presence of antibodies directed toward donor PVG cells was tested in an indirect immunofluorescence assay. Results from sera of allografted rats are shown in Table 1. In contrast to the differences found between the two treatment groups in CTL activity, a significant difference was detected only on POD 17 ( $P < 0.01$ ). At that postoperative time point a higher percentage of alloantibodies was circulating in the blood of untreated allografted rats.

### Complement-Dependent Cytotoxic Activity against Donor Antigens in Recipients of Allografts

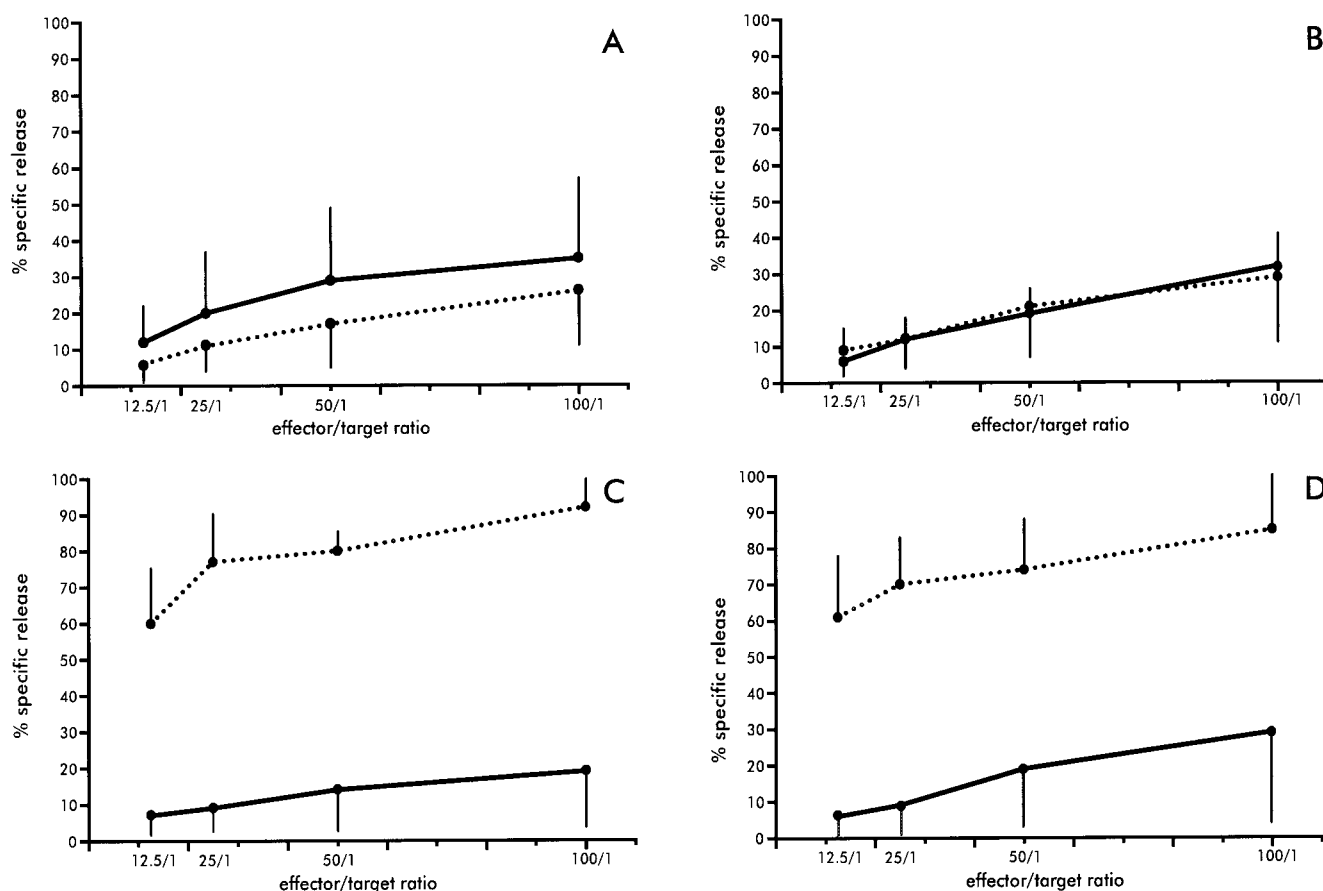
To determine whether there was a difference in the presence of complement-dependent cytotoxic antibodies between the two groups, particularly on POD 17, chromium-release assays were performed. Four serum dilutions at the four different time points were tested. As was to be expected, no detectable cytotoxic activity was found in any of the serum samples, in clodronate liposome-treated rats, or in untreated rats on PODs 3, 7, and 12. Moreover, on POD 17, no cytotoxic antibodies were detectable in the serum of untreated allografted rats. The results obtained for the serum dilution 1:10 are summarized in Table 1.

### Effect of Subconjunctival Administration of Clodronate Liposomes on Immune Responses to Intraperitoneally Injected Allogeneic Lymphocytes

After intraperitoneal injections of PVG lymphocytes, high CTL responses were detected in SLNs and MLNs in untreated and clodronate liposome-treated rats (Table 2). Also, the generation of complement-dependent antibodies was not downregulated by subconjunctival injections of clodronate liposomes in systemically immunized rats (Table 3).

## DISCUSSION

It has been shown that subconjunctival administration of clodronate liposomes after orthotopic corneal allotransplantation prevents graft rejection beyond 100 days in high-risk rats.<sup>10</sup> This *in vivo* depletion of macrophages after transplantation revealed that the presence of macrophages is mandatory for



**FIGURE 2.** Effect of clodronate liposome treatment on CTL responses in the SLNs on POD 3 (A); POD 7 (B); time of onset of graft rejection in the untreated allografts, POD 12 (C); and POD 17 (D). Values represent the mean percentage of specific release  $\pm$ SD of five to seven rats per group. The clodronate liposome-treated group (solid line) was compared with the untreated allografted group (dotted line) at the effector-to-target ratio of 100:1. A significant difference was observed at POD 12 ( $P = 0.006$ ) and POD 17 ( $P = 0.008$ ). The same procedure was followed for the assays described in Figures 3 and 4.

the rejection process. It is unclear, however, where in this process the clodronate liposomes are interfering.

The present study determined the influence of macrophage depletion on the presence of CTLs and antibodies early after transplantation. In the first week after transplantation, no significant difference was found between the two treatment groups, but when the onset of graft rejection occurred in the untreated rats (on POD 12) and on POD 17, the CTL activity detected locally and systemically was significantly less in the treated group. Generation of antibodies also seemed to be impaired in macrophage-depleted rats.

In our model, graft rejection seemed to correlate with increase of CTL activity in the local lymph nodes and the spleen on POD 12 and 17. Although corneal graft rejection is possible in the absence of CD8<sup>+</sup> CTLs,<sup>14,15</sup> it has been shown that rejection can be accompanied by high CTL responses in local lymph nodes or spleen.<sup>16-18</sup> These CTL activities are detected by an *in vitro* assay. How these specific CTLs directed toward donor antigens are induced remains speculative, because corneal grafts are thought to be free of antigen-presenting cells. Therefore, the direct route seems improbable. The generation of CTL activity probably occurs mostly through the indirect pathway. It has been shown that preculturing is mandatory to measure CTL responses in these rats.<sup>18</sup>

Van der Veen et al.<sup>18</sup> tested the same locations for presence of cytotoxic activity toward donor antigens in macrophage-depleted rats after the onset of rejection had occurred. Although no difference was detected in local CTL activity, macrophage depletion downregulated CTL activity in the MLNs as well as the spleen. The results, obtained from both local and more remote lymph nodes, are in slight contrast with findings in the current study. Also the difference found between the two groups was more pronounced in the current study. A possible explanation for these findings may be the use of a different rat strain combination and/or the induction of keratitis before transplantation by van der Veen et al. The eight clodronate liposome-treated allogeneic grafts performed in our experiment 1 demonstrated that administration of clodronate liposomes is also effective in prolonging graft survival in this rat strain combination for at least 100 days. Although the CTL response was more vigorous in these untreated rats, it was still downregulated and graft rejection prevented by macrophage depletion. Early rejection after small bowel allotransplantation is also characterized by a massive influx of macrophages.<sup>19</sup> In this type of solid graft rejection the macrophages seem to be mandatory as well, because intraperitoneal injection of clodronate liposomes prolonged graft survival.<sup>20</sup> Although the precise role of the macrophage in the graft rejection

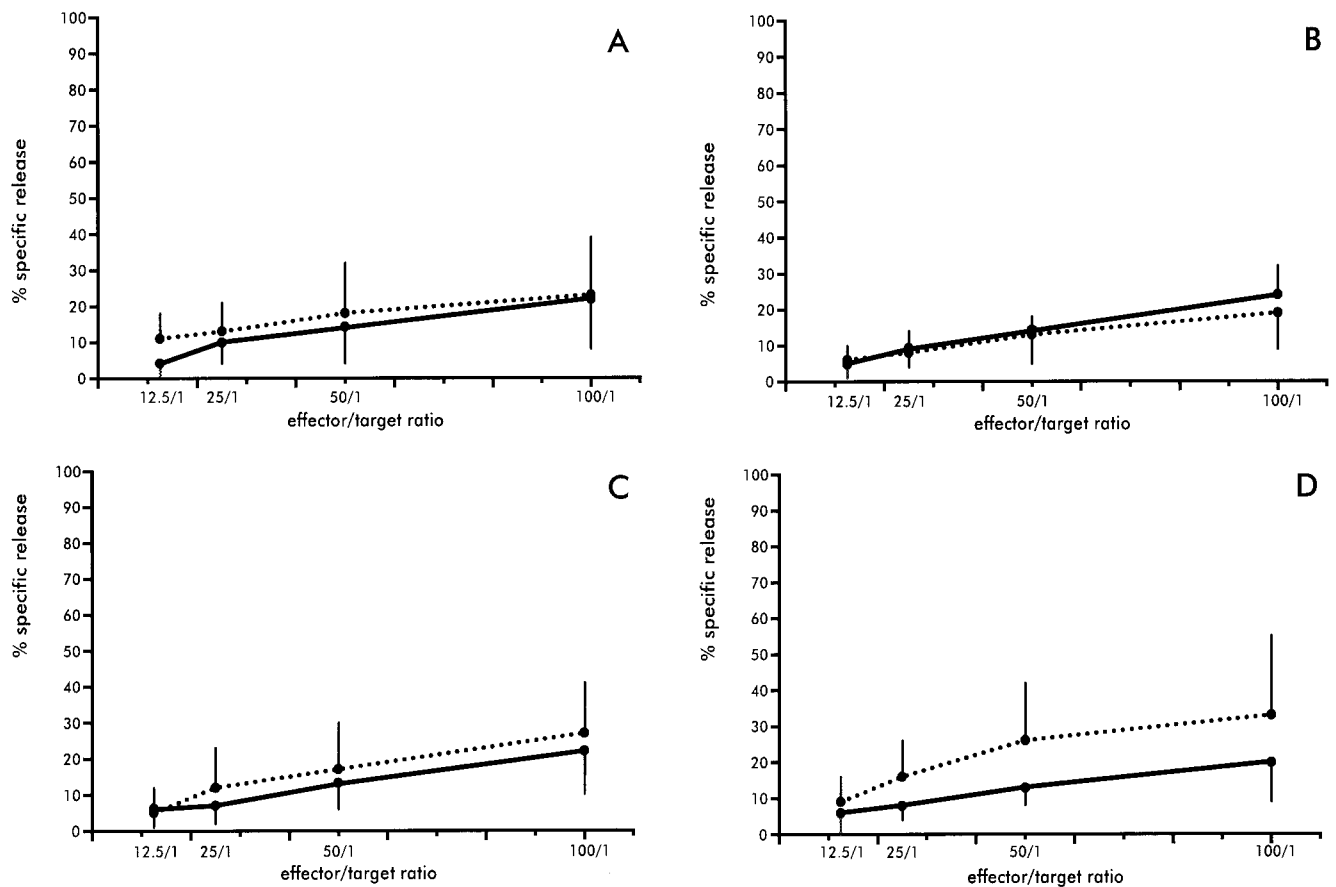


FIGURE 3. Effect of clodronate liposome treatment on CTL responses in MLNs. No significant difference was observed.

tion process is unknown, their presence is required for inducing donor-directed cytotoxicity responses by intestinal epithelial cells.<sup>21</sup>

Another effect of the clodronate liposomes was inhibition of antibody production. Our model showed that in untreated rats, antibodies against donor antigens were detectable 5 days after the onset of graft rejection using an immunofluorescence assay. Unfortunately, it was not possible technically to perform an antibody-dependent cell-mediated cytotoxicity (ADCC) assay, but because the results of the chromium-release assay show that these antibodies were not complement dependent, these results indirectly suggest that they may be cell-dependent cytotoxic antibodies. Van der Veen et al.<sup>18</sup> also showed that macrophage depletion impairs generation of complement-dependent antibodies after corneal transplantation. Lung allotransplantation leads to production of both types of antibodies. Depleting donor macrophages before transplantation by intratracheal instillation of clodronate liposomes does not prolong graft survival, but the antibodies involved, both in ADCC and complement-dependent cytotoxicity are significantly decreased.<sup>22</sup> Also the presence of these antibodies is found in chronic rejection of cardiac allografts and is related to activation of macrophages.<sup>23</sup>

As mentioned earlier, corticosteroids have been found to be very effective in preventing graft rejection, but aside from the fact that not all rejections can be inhibited, this drug also has many side effects. In the attempt to find new therapeutic

regimens, an important goal is the absence of systemic side effects. A major concern about the mechanism by which clodronate liposomes work could be that macrophage depletion was accomplished not only locally but also systemically. Experiment 3 showed that subconjunctival injection of clodronate-containing liposomes, using the same procedure as was used after corneal transplantation, did not interfere with the cellular and humoral immune response in rats injected intraperitoneally with allogeneic lymphocytes. Probably only the conjunctival macrophages are depleted with this procedure. Van Klink et al.<sup>2</sup> showed that in Chinese hamsters, repeated subconjunctival injections with clodronate liposomes led to depletion of acid phosphatase-positive macrophages in the conjunctiva. By contrast, the number of acid-phosphatase-positive cells in the corneal epithelium, stroma, ciliary body, and iris was not affected.<sup>24</sup> An explanation for this could be that redistribution of liposomes after local injection is hindered by the anatomic features of the cornea.<sup>25</sup>

Another point for concern could be that liposome treatment affects not only the macrophage population but also the dendritic cell population. No experimental studies have been published so far in which the number of dendritic cells or the function of the dendritic cell population is measured after subconjunctival injections of clodronate liposomes. It has been shown, however, that after intravenous injections of clodronate liposomes the number and distribution of dendritic cells in the spleen is not affected. Also the antigen-presenting

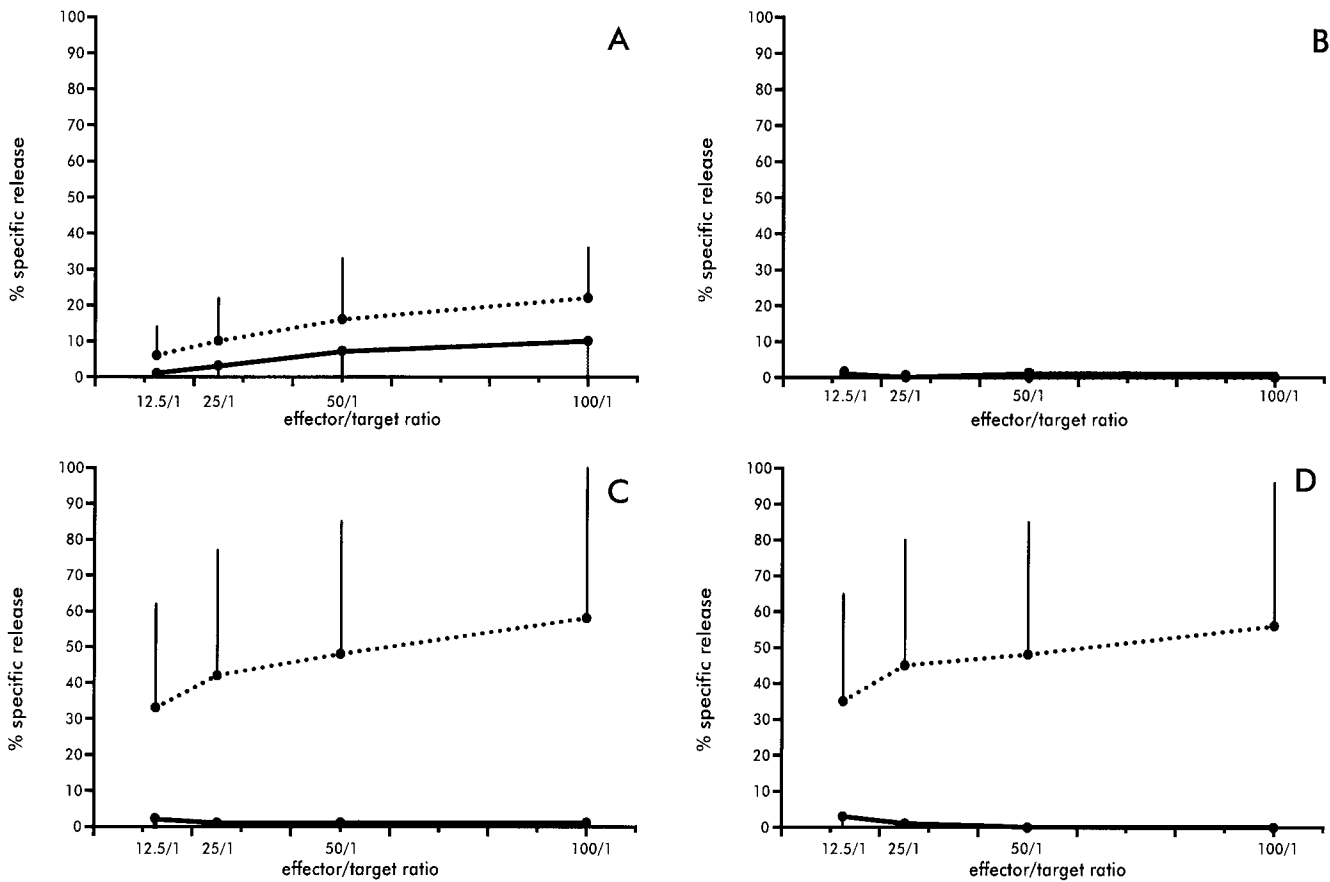


FIGURE 4. Effect of clodronate liposome treatment on CTL responses in spleen. A significant difference was observed at POD 12 ( $P = 0.008$ ) and POD 17 ( $P = 0.009$ ).

cell activity of splenocytes and lymph node cells, measured by in vitro primary CTL responses to ovalbumin or mixed lymphocyte reactions to allogeneic antigens, were comparable in clodronate liposome-treated and untreated mice.<sup>26,27</sup> After intraperitoneal injections with clodronate liposomes, these mice showed similar flow cytometry analysis data for dendritic cells as the control PBS-liposome treated group.<sup>28</sup>

Using the corneas from the rats used in this study, Torres et al.<sup>29</sup> determined the effect of administration of local clodronate liposome on cytokine mRNA expression within the cornea. In the macrophage-depleted rats, decreased expression of interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, interferon (IFN)- $\gamma$ , and

tumor necrosis factor (TNF)- $\beta$ /lymphotoxin (LT) mRNA was found. Treatment with clodronate liposomes after corneal grafting thus resulted in decreased local mRNA expression of several macrophage-derived cytokines and T-cell derived cytokines and reduced levels of CTLs and antibodies.

The mechanism by which the clodronate liposomes induce immunosuppression remains highly speculative. From the fact that clodronate liposomes interfere with only the macrophage population, several hypotheses can be proposed. Because no effector mechanisms, such as CTLs and antibody production, are present, it could be concluded that there is an interruption in the afferent arc of the immune response leading

TABLE 1. Effect of Clodronate Liposomes on Humoral Immune Response after Orthotopic Transplantation

	POD 3		POD 7		POD 12		POD 17	
	LIP	Untreated	LIP	Untreated	LIP	Untreated	LIP	Untreated
Antibodies*	2 $\pm$ 2	2 $\pm$ 3	2 $\pm$ 3	2 $\pm$ 2	3 $\pm$ 1	4 $\pm$ 1	3 $\pm$ 3 $\ddagger$	36 $\pm$ 12
Complement-dependent antibodies $\dagger$	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 1	0 $\pm$ 0	1 $\pm$ 1	0 $\pm$ 0	2 $\pm$ 1	3 $\pm$ 5

Alloantibody response, toward PVG lymphocytes, in AO rats after orthotopic allotransplantation. Clodronate liposome-treated rats (LIP) are compared with untreated rats at different PODs for the serum dilution 1:10. Values are presented as mean  $\pm$  SD.

\* Immunofluorescence assay (percentage positive staining cells).

$\dagger$  Chromium release assay (percentage specific <sup>51</sup>Chromium release).

$\ddagger P < 0.01$ .



TABLE 2. Effect of Subconjunctival Administration of Clodronate Liposomes on Systemic Cellular Immune Responses

Effector-to-Target Ratio	SLN		MLN		Spleen	
	Cl <sub>2</sub> MDP-LIP-Treated	Untreated	Cl <sub>2</sub> MDP-LIP-Treated	Untreated	Cl <sub>2</sub> MDP-LIP-treated	Untreated
12.5:1	43 ± 31	28 ± 9	6 ± 10	20 ± 20	0 ± 0	8 ± 10
25:1	67 ± 27	49 ± 4	15 ± 14	27 ± 13	2 ± 4	3 ± 4
50:1	82 ± 20	63 ± 11	31 ± 20	44 ± 10	8 ± 12	4 ± 6
100:1	100 ± 3*	87 ± 4	52 ± 17*	70 ± 11	14 ± 19*	10 ± 13

Cell-mediated immune responses in AO rats immunized intraperitoneally with PVG lymphocytes. The rats were killed 10 days after immunization. Values are presented as mean percentage specific <sup>51</sup>chromium release ± SD. The Cl<sub>2</sub>MDP-liposome (LIP)-treated group was compared with the untreated group by Mann-Whitney test for the 100:1 ratio.

\*  $P > 0.05$ .

to graft rejection. Macrophages can display a variety of functions that can be immunosuppressive or stimulatory. They are able to present antigen, but less so than dendritic cells or Langerhans' cells.<sup>27</sup> Our hypothesis is that macrophages interact with dendritic cells, and the antigen-presenting function of this latter cell is therefore enhanced. Interaction of lung macrophages with dendritic cells to present antigenic peptides has been reported. Depending on the ratio of interstitial macrophages to dendritic cells, the dendritic cell function is either augmented or inhibited.<sup>30</sup> Alternatively, macrophages can mediate with the influx of T cells through upregulation of adhesion molecules or production of cytokines by the macrophage population, T cells are attracted to enter the inflammatory site. Fox et al.<sup>26</sup> showed that macrophage depletion after xenografting in nonobese diabetic mice leads to reduced graft infiltration by T lymphocytes and eosinophils without directly affecting the function of the T cells or the dendritic cells. Van der Veen et al.<sup>18</sup> also showed an decreased influx of immunocompetent cells into the graft of clodronate-treated rats after corneal allotransplantation and the expression of intercellular adhesion molecule (ICAM)-1 and leukocyte function-associated antigen (LFA)-1 is significantly reduced in these clodronate liposome-treated rats compared with untreated rats.<sup>18,31</sup>

At this moment a study is in progress to determine the delayed-type hypersensitivity responses of clodronate-treated animals after orthotopic corneal allotransplantation. Results of this study will resolve the question of whether antigen presentation has occurred and the effector phase of rejection has started in these clodronate liposome-treated animals.

TABLE 3. Effect of Subconjunctival Administration of Clodronate Liposomes on Systemic Humoral Immune Responses

Serum Dilution	Cl <sub>2</sub> MDP-LIP-Treated	Untreated
1:80	38 ± 27	15 ± 21
1:40	37 ± 32	17 ± 24
1:20	49 ± 28	34 ± 25
1:10	60 ± 12*	38 ± 2

Alloantibody response in AO rats immunized intraperitoneally with PVG lymphocytes. The rats were killed 10 days after immunization. Values are presented as mean percentage specific <sup>51</sup>chromium release ± SD. The Cl<sub>2</sub>MDP-liposome (LIP)-treated group was compared with the untreated group by Mann-Whitney test for the 1:10 dilution.

\*  $P > 0.05$ .

Our study shows that after corneal allotransplantation in untreated rats, high CTL responses are detected locally and systemically, as well as complement-independent antibodies at time of rejection. We conclude that macrophage depletion, through repeated subconjunctival injections of clodronate liposomes, downregulates these high CTL responses and impairs antibody formation.

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